

Applicants: Jay M. Short
Application No.: 09/753,752
Filed: January 2, 2001
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In the Specification

Please amend the paragraph beginning on page 34 at line 17 as follows:

Re-Blunt DNA

- B'
1. pool all left over DNA that was not ligated to the lambda arms
(Fractions 1-7) and add H₂O to a final volume of 12 µl. Then
add :

143 µl	H ₂ O
20 µl	10X Buffer 2 (from Stratagene <u>STRATAGENE®</u> 's cDNA Synthesis Kit)
23 µl	Blunting dNTP (from Stratagene <u>STRATAGENE®</u> 's cDNA Synthesis Kit)
2.0 µl	Pfu (from Stratagene <u>STRATAGENE®</u> 's cDNA Synthesis Kit)
 2. Incubate 72 °C, 30 minutes.
 3. Phenol/chloroform extract once.

Please amend two paragraphs beginning on page 35 at line 10 as follows:

Adaptor Ligation

- B²
1. Gently resuspend DNA in 8 µl Eco RI adaptors (from ~~Stratagene~~
STRATAGENE®'s cDNA Synthesis Kit).
 2. Add:

1.0 µl	10X Ligation Buffer
1.0 µl	10 mM rATP
1.0 µl	T4 DNA Ligase (4Wu/ µl)
 3. Incubate 4° C/ 2 days.

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(Do NOT cut back since using ADAPTORS this time. Instead, need to ~~phospherylate~~
phosphorylate)

Phosphorylate Adaptors

- B3
cont
1. Heat kill ligation reaction 70° C, 30 minutes.
 2. Add:

1.0 µl	10X Ligation Buffer
2.0 µl	10mM rATF
6.0 µl	H ₂ O
1.0 µl	PNK (from Stratagene <u>STRATAGENE</u> ®'s cDNA Synthesis Kit).
 3. Incubate 37° C, 30 minutes.

Please amend the paragraph at page 38, line 6 as follows:

B3

Cell collection and preparation of DNA. Agarose plugs containing concentrated picoplankton cells were prepared from samples collected on an oceanographic cruise from Newport, Oregon to Honolulu, Hawaii. Seawater (30 liters) was collected in Niskin bottles, screened through 10 µm ~~Nitex~~ NITEXTM filter, and concentrated by hollow fiber filtration (~~Amicon~~ AMICONTM DC10) through 30,000 MW cutoff polysulfone filters. The concentrated bacterioplankton cells were collected on a 0.22 µm, 47 mm ~~Durapore~~ DURAPORE® filter, and resuspended in 1 ml of 2X STE buffer (1M NaCl, 0.1M EDTA, 10 mM Tris, pH 8.0) to a final density of approximately 1 X 10¹⁰ cells per ml. The cell suspension was mixed with one volume of 1% molten ~~Seaplaque~~ SEAPLAQUE® LMP agarose (FMC) cooled to 40°C, and then immediately drawn into a 1 ml

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B3
cont.
syringe. The syringe was sealed with parafilm and placed on ice for 10 min. The cell-containing agarose plug was extruded into 10 ml of Lysis Buffer (10 mM Tris pH 8.0, 50 mM NaCl, 0.1M EDTA, 1% Sarkosyl, 0.2% sodium deoxycholate, a mg/ml lysozyme) and incubated at 37°C for one hour. The agarose plug was then transferred to 40 mls of ESP Buffer (1% Sarkosyl, 1 mg/ml proteinase-K, in 0.5M EDTA), and incubated at 55°C for 16 hours. The solution was decanted and replaced with fresh ESP Buffer, and incubated at 55°C for an additional hour. The agarose plugs were then placed in 50 mM EDTA and stored at 4°C shipboard for the duration of the oceanographic cruise.

Please amend the paragraph beginning at page 39, lines 26 as follows:

B4
Vector arms are prepared from pFOS1 as described (Kim *et al.*, Stable propagation of cosmid sized human DNA inserts in an f-factor based vector, *Nucl. Acids Res.*, 20:10832-10835, 1992). Briefly, the plasmid is completely digested with *AstII*, dephosphorylated with *HK* phosphatase, and then digested with *BamHI* to generate two arms, each of which contains a *cos* site in the proper orientation for cloning and packaging ligated DNA between 35-45 kbp. The partially digested picoplankton DNA is ligated overnight to the pFOS1 arms in a 15 µl ligation reaction containing 25 ng each of vector and insert and 1U of T4 DNA ligase (~~Boehringer Mannheim~~ BOEHRINGER MANNHEIM CORP®). The ligated DNA in four microliters of this reaction is *in vitro* packaged using the ~~Gigapack~~ GIGAPACK® XL packaging system (STRATAGENE® INC.), the fosmid particles transfected to *E. coli* strain DH10B (BRL), and the cells spread onto LB_{cm15} plates. The resultant fosmid clones are picked into 96-well microliter dishes containing LB_{cm15} supplemented with 7% glycerol. Recombinant fosmids, each containing ca. 40 kb of picoplankton DNA insert, have yielded a library of 3.552 fosmid clones,

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B4
cont. containing approximately 1.4×10^8 base pairs of cloned DNA. All of the clones examined contained inserts ranging from 38 to 42 kbp. This library is stored frozen at -80°C for later analysis.

Please amend the paragraph beginning on page 41 at line 5 as follows:

Screening for Tier 1-hydrolase: Tier 2-amide

BS The eleven plates of the Source Library were used to multiply inoculate a single plate (the "Condensed Plate") containing in each well 200 μL of LB Amp/Meth, glycerol. This step was performed using the High Density Replicating Tool (HDRT) of the ~~Beckman-Biomek~~ BECKMAN BIOMEK® device with a 1% bleach, water, isopropanol, air-dry sterilization cycle in between each inoculation. Each well of the Condensed Plate thus contained 11 different ~~pBluescript~~ pBLUESCRIPT® cloning vector clones from each of the eleven source library plates. The Condensed Plate was grown for 2h at 37°C and then used to inoculate two white 96 well ~~Dynatech~~ DYNATECH® microtiter daughter plates containing in each well 250 μL of LB amp/Meth, glycerol. The original condensed ~~plates~~ plate was incubated at 37°C for 18h then stored at -80°C . The two condensed daughter plates were then heated at 70°C for 45 min. to kill the cells and inactivate the host E. coli enzymes. A stock solution of 5mg/mL morphourea phenylalanyl-7-amino-4-trifluoromethyl coumarin (MuPheAFC, the 'substrate') in DMSO was diluted to 600 μM with 50 mM pH 7.5 Hepes buffer containing 0.6 mg/mL of the detergent dodecyl maltoside.
